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Germ Cell-Specific Expression of Microphthalmia-Associated Transcription Factor mRNA in Mouse Testis

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The gene coding for microphthalmia-associated transcription factor (Mitf) contains many promoters that could generate multiple Mitf isoforms with distinct amino-termini, such as ubiquitously expressed Mitf-A and Mitf-H. To gain further insight into Mitf isoform multiplicity and the regulation of the promoter usage of the *Mitf* gene, we have analyzed the function of the amino-terminal domains of Mitf isoforms and the expression of Mitf mRNA in mouse postnatal testis, which is characterized by spermatogenesis and by a cool temperature because of its unique location. Here we show that the amino-terminal domain of Mitf-A possesses a transactivation activity, as judged by yeast expression analysis. We also show the expression of Mitf-A and Mitf-D mRNAs in testis by PCR-based methods. Moreover, *in situ* hybridization analysis revealed that an Mitf mRNA, probably representing Mitf-A and/or Mitf-D, is expressed in germ cells, including spermatogonia, spermatocytes that undergo meiosis, and round spermatids with the haploid genome, but is undetectable in elongated spermatids with remodeled and condensed chromatin. Notably, Mitf mRNA is undetectable in somatic Leydig cells and peritubular cells. Therefore, multiple promoters may direct differential expression of the *Mitf* gene in the testis and contribute to functional diversity of Mitf isoforms.

Key words: gene transcription; germ cell; microphthalmia; spermatogenesis; testis.

Abbreviations: Mitf, microphthalmia-associated transcription factor; bHLH-LZ, basic helix-loop-helix-leucine zipper; RPE, retinal pigment epithelium; RT, reverse transcription.

Microphthalmia-associated transcription factor (Mitf) is important in the development and differentiation of neural crest-derived melanocytes, bone marrow-derived mast cells and osteoclasts, and optic cup-derived retinal pigment epithelium (RPE) (*1–4*). Mitf belongs to a family of transcription factors that contain a basic helix-loop-helix and leucine-zipper (bHLH-LZ) structure (*1*, *2*). Mitf comprises many isoforms with unique amino-termini, such as Mitf-M, Mitf-A, Mitf-H, Mitf-D, and Mitf-E (*5–8*), which share the entire downstream region, including the bHLH-LZ domain (see Fig. 1A). Mitf-M is exclusively expressed in melanocytes and melanoma cells and is under the regulation of the melanocyte-specific promoter (*M* promoter) (*9*), whereas other isoforms are expressed in many cell types. Importantly, Mitf has been identified as a new class of nuclear mediators of Wnt signaling, which is important in the differentiation of neural crest cells (*10–12*).

Among many *Mitf* mutant mice, we are interested in the two recessive mutants, red-eyed white $Mitf^{mi\text{-}rw}$ (*4*) and black-eyed white $Mitf^{mi\text{-}bw}$ (*13*), both of which are associated with large structural changes in the *Mitf* gene (*14*, *15*) (see Fig. 1A). Homozygous $Mitf^{mi\text{-}rw}$ mice exhibit small red eyes and a white coat (*3*). Homozygous $Mitf^{mi\text{-}bw}$ mice exhibit complete white coat color and deafness due to the lack of melanocytes, but normally pigmented RPE (*15*, *16*). The molecular lesion in $Mitf^{mi\text{-}rw}$ mice is the deletion of a large DNA segment that includes exon 1H, exon 1D, and exon B1b (*14*, *17*). Thus, the $Mitf^{mi\text{-}rw}$ mice lack authentic Mitf isoforms, except for Mitf-M, because exon 1M is located far downstream (6 kb) from the deletion and its transcription is not completely repressed (*14*, *17*). Importantly, aberrant Mitf mRNA is over-expressed in the testes of $Mitf^{mi\text{-}rw}$ mice (*3*), suggesting that the deleted genomic DNA segment may contain the regulatory elements that repress the transcription of the *Mitf* gene in the testis. In contrast, the molecular lesion in $Mitf^{mi\text{-}bw}$ is the insertion of an L1 retrotransposable element in intron 3 between exon 3 and exon 4, which leads to repression of Mitf-M mRNA expression and a loss of melanocytes (*15*). Incidentally, the difficulty in obtaining a large numbers of $Mitf^{mi\text{-}bw}$ mouse offspring was experienced (*15*), although normal fertility was described in the original report (*13*).

Much attention has been given to the potential interaction between the *Mitf* allele and the white spotting (*W*) allele that codes for c-kit trans-membrane tyrosine

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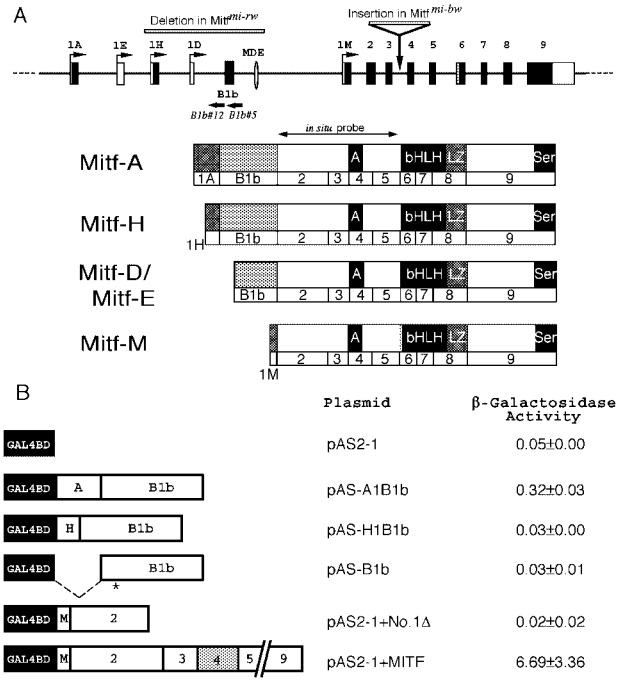

Fig. 1. **Multiple Mitf isoforms encoded by the single *Mitf* gene.** A: Schematic representation of the mouse *Mitf* gene and Mitf isoforms. The direction of transcription is from left to right, as indicated by the arrows. The 5′- and 3′-untranslated exons are shown as open boxes, and the protein-coding regions are shown as closed boxes. The numbers, shown above the boxes or below the Mitf isoforms, indicate exons. The N-termini of the isoforms are encoded by exon 1A, exon 1H, exon B1b or exon 1M. Mitf-D and Mitf-E share an identical primary structure. Exon B1b is used as a second exon for the generation of Mitf-A, Mitf-D, Mitf-E, and Mitf-H mRNAs. MDE represents an upstream enhancer of the *M* promoter (*17*). Also indicated are the positions of the deletion identified in *Mitf*$^{mi\text{-}rw}$ mice (*14*) and the insertion in *Mitf*$^{mi\text{-}bw}$ mice (*15*). Exons 2 to 9 are common to all isoforms that share the transcriptional activation domain (A) (*28*), the bHLH-LZ structure, and the serine-rich region (Ser). The position of the cRNA probe used for *in situ* hybridization (Fig. 4) is also shown. B: Functional analysis of the amino-terminal regions of MITF isoforms in yeast cells. The structures of fusion proteins, consisting of the GAL4 DNA-binding domain (GAL4 BD) and the amino-terminal region of a given MITF isoform, are schematically shown. The amino-terminus of Mitf-D is located in the B1b domain, shown as an asterisk. A transcriptional activation domain is encoded by exon 4 (*28*), shown as a shaded box. The results of at least three independent experiments are shown with standard deviations.

kinase receptor (*18*, *19*). Like *Mitf* mutants, *W* mutants show a deficiency in melanocytes and mast cells, whereas, unlike *Mitf* mutants, *W* mutants also show a deficiency in germ cells. In fact, mast cells of the classic *Mitf*^{mi} mutant display low numbers of c-kit receptors (*20*), and Mitf transactivates the c-*kit* gene promoter in cultured murine mast cell lines (*21*). Thus, Mitf may be important in the regulation of c-kit expression in mast cells. In the testis, c-kit is expressed on spermatogonia, early spermatocytes, and Leydig cells (*22*), but there are no reports concerning Mitf expression in the testis. We are therefore interested in the transcriptional regulation of the *Mitf* gene in the postnatal testis, which is characterized by spermatogenesis and by a cool temperature of 33°C because of its unique location in the scrotum as compared with other internal organs at 37°C.

Spermatogenesis is the highly ordered process that consists of mitosis, meiosis, and the post-meiotic phase, and depends on well-orchestrated programs that control cellular differentiation in the seminiferous tubules of the testis (*23*, *24*). Spermatogonial stem cells adjacent to the basal membrane of seminiferous tubules undergo mitotic division not only to renew the population of stem cells but also to generate spermatogonia that eventually develop into spermatozoa (sperm). Notably, more than half of spermatogonia undergo apoptosis. During spermatogenesis, diploid spermatogonia differentiate into spermatocytes that undergo meiosis, thereby generating haploid spermatids. Subsequently, spermatids differentiate into mature spermatozoa during spermiogenesis.

Here we have analyzed the function of the amino-terminal domains of Mitf isoforms that are encoded by separate exons of the *Mitf* gene. In addition, we have analyzed the expression of Mitf mRNA in postnatal mouse testes by *in situ* hybridization and real-time RT-PCR analyses to gain insight into the regulation of the promoter selection of the *Mitf* gene.

EXPERIMENTAL PROCEDURES

Transactivation Assay of MITF Domains in Yeast— The transactivation activity of the amino-terminal regions of MITF isoforms was assessed using MATCHMAKER two-hybrid system 2 (Clontech). The cDNA segment for each amino-terminal region was prepared from MITF-A or MITF-H cDNA (*5*) by PCR. The primers used were *MIApr2* (positions 37 to 57 of MITF-A cDNA) and *3′H2* (5′-CTTAAGGACTTCCATCGGCACCTG-3′) for domains A and B1b, primers *5′H2* (5′-AGTTCCGCCGAGCATCCTGGG-3′) and *3′H2* for domain B1b, and primers *5′H1* (5′-GCAGAACACCTTAAAGGAAAA-3′) and *3′H2* for domains H and B1b (*5*). Each target fragment was cloned in the multi-cloning site of pAS2-1, encoding a GAL4 DNA-binding domain, to maintain the correct reading frame. The resulting plasmids were designated pAS-A1B1b, pAS-B1b, and pAS-H1B1b, coding for the amino-terminal of MITF-A, MITF-D, and MITF-H, respectively (see Fig. 1B). Fusion proteins encoded by pAS-A1B1b and pAS-H1B1b contain 16 and 8 amino acid residues as linker seqeunces, respectively. The 5′ region of the MITF-M cDNA was amplified by PCR from a subclone phMI-9 carrying a full-length MITF-M cDNA (*25*). The primers used were 5′-ggatccATGCTGGAAATGCTAGAATA-3′ (positions 121 to 140 and an additional *Bam*HI site at the 5′ side of the first Met codon) (*9*) and 5′-GTCATCGATTACATCATCCA-3′ (complementary to the positions 467 to 486; the native *Cla*I site underlined). The amplified fragment was digested with *Bam*HI and *Cla*I, and the resulting fragment was ligated together with the *Cla*I–*Eco*RI fragment (positions 480 to 1623) derived from phMI-9 to pAS2-1, which was linearized with *Bam*HI and *Eco*RI. The resulting plasmid, pAS2-1+MITF, contains the full-length MITF-M cDNA. For the amino-terminus of MITF-M, pGEX-MITF (*26*) was digested with *Bam*HI and *Nco*I, and the cDNA fragment coding for the region from Met 1 to Met 75 was ligated to the multi-cloning site of pAS2-1, yielding pAS2-1+No.1Δ. Transformation of yeast Y187 strain with each plasmid and liquid culture assay us-

ing *o*-nitrophenyl β-D-galactopyranoside were performed according to the manufacturer's instructions (Clontech).

Mice—The original mutant strain (C3HBSt) carrying the *Mitf*$^{mi\text{-}bw}$ allele was kindly provided by Dr. Walter C. Quevedo Jr. (Brown University, Providence, RI), and maintained on a C3H background at Tohoku University, Biological Institute, as detailed previously (*15*). In this study, tissues were isolated from C3H/HeSlc mice, purchased from Japan SLC (Hamamatsu), and *Mitf*$^{mi\text{-}bw}$ mice.

Isolation of the 5′-Ends of Mitf Isoform mRNA—Total RNA was extracted from the testes of C3H mice (8 wk old) by the guanidium thiocyanate-cesium chloride method. The 5′ ends of Mitf cDNA were cloned from testicular RNA by rapid amplification of 5′ cDNA end (5′-RACE) using a Marathon cDNA Amplification Kit (Clontech). RNA was subjected to amplification by PCR with an adapter primer 1 and a reverse primer B1b5 (5′-GTTGCTGGCGTAGCAAGATGCGTGATGTCA-3′), and by a second PCR with an adapter primer 2 and a reverse primer B1b12 (5′-AGGAGCTTATCGGAGGCTTGGAGGCCCCAG-3′) (see Fig. 1A). These two reverse primers correspond to positions 54–83 and positions 18–47 of the cDNA segment coding for domain B1b (referred to exon B1b), respectively (*7*). The PCR conditions used were 5 cycles of 96°C for 20 s and 72°C for 1 min, 5 cycles of 96°C for 20 s and 70°C for 1 min, and then 25 cycles of 96°C for 20 s and 68°C for 1 min. The amplified DNA fragments were subjected to direct sequencing using the adapter primer 2 or the B1b12 primer. The amplified DNA fragments were also subcloned into pGEM-T vector (Promega), and the nucleotide sequences of the cloned DNA fragments were determined in both directions using a 377A autosequencer (Perkin-Elmer Biosystems).

Real-Time RT-PCR—Real-time RT-PCR analysis was performed using a TaqMan Core Reagent Kit and an ABI Prism 7700 Sequence Detection System (Perkin-Elmer Biosystems). Each PCR primer set was designed to amplify a given exon 1 sequence. The forward and reverse primers for exon 1M (Mitf-M) were (57–81; 5′-TACAGAAAGTAGAGGGAGGAGGACT-3′) and (106–122; 5′-CTTGGGGCTGCCTGAAA-3′), respectively (*3*). Likewise, the forward and reverse primers for exon 1A (Mitf-A) were (123–139; 5′-TGCAGTCCGAATCGGGA-3′) and (174–197; 5′-ACGAAGAACCCAAAACCTATTACG-3′), and those for exon 1H (Mitf-H) were (30–52; 5′-GCGCTTAGATTTGAGATGCTCAT-3′) and (90–107; 5′-GAGCATTCTGGGGCCTCC-3′) (*5*). The TaqMan probes were (83–104; 5′-AGTGGTCTGCGGTGTCTCCTGG-3′) for exon 1M, (141–162; 5′-TCGTGGCGGATTTCGAAGTCGG-3′) for exon 1A, and (54–82; 5′-CCCTGCTCCTTTGAAAGCTTGTGTCTCAG-3′) for exon 1H, labeled with reporter dye (FAM) at the 5′ end and with quencher dye (TAMRA) at the 3′ end. TaqMan Ribosomal RNA Control Reagent (Perkin-Elmer Biosystems) was used as an endogenous control to normalize the RNA quantity. PCR conditions were as follows: 10 μl RNA solution was added to 40 μl reaction mixture containing TaqMan Buffer A, 5.5 mM $MgCl_2$, 0.3 μM dATP, 0.3 μM dCTP, 0.3 mM dGTP, 0.6 mM dUTP, 0.2 μM each primer, 0.1 μM each TaqMan probe, 25 mU AmpliTaq Gold DNA polymerase, RNase Inhibitor, and reverse transcriptase (MS-RT). The thermal cycler conditions were 30 min at 48°C for reverse transcription, 10 min at 95°C for activation of AmpliTaq Gold DNA polymerase, and then 40 cycles of 15 s at 95°C (denature) followed by 1 min at 60°C (annealing and extension). Fluorescence intensity was measured with an ABI Prism 7700 Sequence Detection System. Standard curves for Mitf RNAs were generated with templates prepared using Riboprobe® In vitro transcription systems (Promega). The template for Mitf-M RNA was generated from mouse Mitf-M cDNA (positions 11 to 172) by PCR, and subcloned into pGEM-T vector (Promega). For the preparation of Mitf-A RNA, the *Hin*dIII–*Eco*RI fragment of mouse Mitf-A cDNA (*15*) was subcloned into BluescriptII SK+, and for the Mitf-H RNA, the PCR fragment (positions 30 to 107) of Mitf-H cDNA was subcloned into pGEM-T Easy vector (Promega). Standard curves for ribosomal RNA were generated using total RNA prepared from B16-F1 mouse melanoma cells. The threshold cycle (Ct) value was used to plot a standard curve in which Ct decreased in linear proportion to the log of the change in normalized reporter fluorescence (ΔRn).

In Situ Hybridization Analysis—The testes of adult mice (8 wk old) were isolated, and the tunica albuginea was dissected to assure proper fixation. The dissected testes were fixed for 16 h in phosphate buffer containing 4% paraformaldehyde (PFA), 0.5% glutaraldehyde, 1 mM $MgCl_2$ and 1 mM $CaCl_2$ (*27*). After fixation, the tissues were dehydrated and embedded in OCT compound (Miles Inc.). Sections (8 μm thickness) were cut at –20°C, collected on silanized slides and stored at –70°C. The sections were pretreated by microwave heating at 95°C for 15 min and incubated with 1 μg/ml proteinase K for 15 min at 37°C. The Mitf cRNA probe corresponds to the common region (positions 151–719 of mouse Mitf-M cDNA) (see Fig. 1A). The probe was synthesized *in vitro* in an antisense orientation by T3 RNA polymerase in the presence and absence of digoxigenin-11-UTG (DIG) using the DIG-labelling system (Boehringer Mannheim) and Riboprobe® In vitro transcription systems (Promega), respectively. The DIG-labeled cRNAs were alkaline-treated so that the average lengths of the probes were 150 bases. To detect low levels of Mitf mRNA in the testis, the DAKO GenPoint™ Catalyzed Signal Amplification System (DAKO) was employed. Hybridization signals were detected using the Digoxigenin (DIG)/HRP F(ab′) [affinity-isolated Rabbit Polyclonal Ab] and the DAKO GenPoint™ Catalyzed Signal Amplification System.

RESULTS

Functional Analysis of the Amino-Terminal Domains of Mitf Isoforms—Mitf isofoms differ at their N-termini, which are encoded by separate first exons, except for exon 1D and exon 1E, which code for the 5′-untranslated regions of their mRNAs (Fig. 1A). Mitf isoforms share the entire C-terminal region encoded by exons 2 to 9, including a transactivation domain (*28*) and a bHLH-LZ structure. Here we focus on the four Mitf isoforms (Mitf-A, -H, -D, and -M) that have been well characterized; these isoforms could function as nuclear mediators of Wnt signaling (*10*, *11*) and are expressed in both human and mouse cells (*2*, *3*, *5*, *7*). The amino-terminus of Mitf-A, domain A, is encoded by exon 1A and consists of 35 amino acid resi-

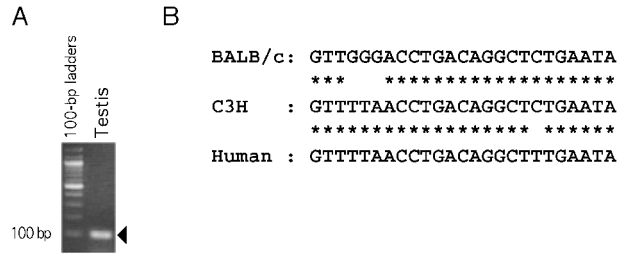

Fig. 2. **Mitf-D as a predominant isoform in the mouse testis.** A: 5′-RACE analysis of Mitf mRNA expression in adult mouse testis. Shown are the amplified 5′-cDNA segments of 104 bp (arrowhead), including the adapter primer of 32 bp. Size markers are the 100-bp DNA ladders. B: The nucleotide sequence of the 5′ portion of the amplified C3H Mitf-D cDNA is aligned with the previously reported sequences of BALB/c mouse and human cDNAs (*7*).

dues (*5*). Likewise, domain H, encoded by exon 1H, consists of 19 amino acids (*5*). Domain A and domain H are followed by domain B1b of 83 amino acids, which is encoded by exon B1b. The first Met of Mitf-D is present in domain B1b, and corresponds to codon 53 of Mitf-A (*7*). Exon 1M is under the regulation of the melanocyte-specific *M* promoter (*9*), and domain M consists of 11 amino acids (*29*).

As a first attempt to assess the function of the unique N-terminal domains, we analyzed the transactivation activity of human MITF isoforms in yeast (Fig. 1B). As a positive control, a construct pAS2-1+MITF, coding for the fusion proteins of the GAL4 DNA-binding domain and entire MITF-M, was included. Expression of this construct resulted in the highest β-galactosidase activity, whereas no transactivation activity was detected with the N-terminal region of MITF-M (Met 1 to Met 75). These results are consistent in part with the notion that the transcriptional activation domain is encoded by exon 4 (*28*), thereby indicating the suitability of the assay method. A significant β-galactosidase activity was detected with construct pAS-A1B1b, which codes for the amino-terminal region of MITF-A (domain A plus domain B1b), but not with domain B1b, which includes the N-terminal region of MITF-D (*7*), or with the N-terminal region of MITF-H (domain H plus domain B1b). These results suggest that domain A may possess the transactivation activity.

Expression of Mitf-D mRNA in the Testis—Mitf mRNA expression was undetectable in mouse testis by Northern blot analysis (data not shown), as reported by other investigators (*3*). Consequently, to identify Mitf isoforms expressed in adult mouse testis, we performed 5′ RACE

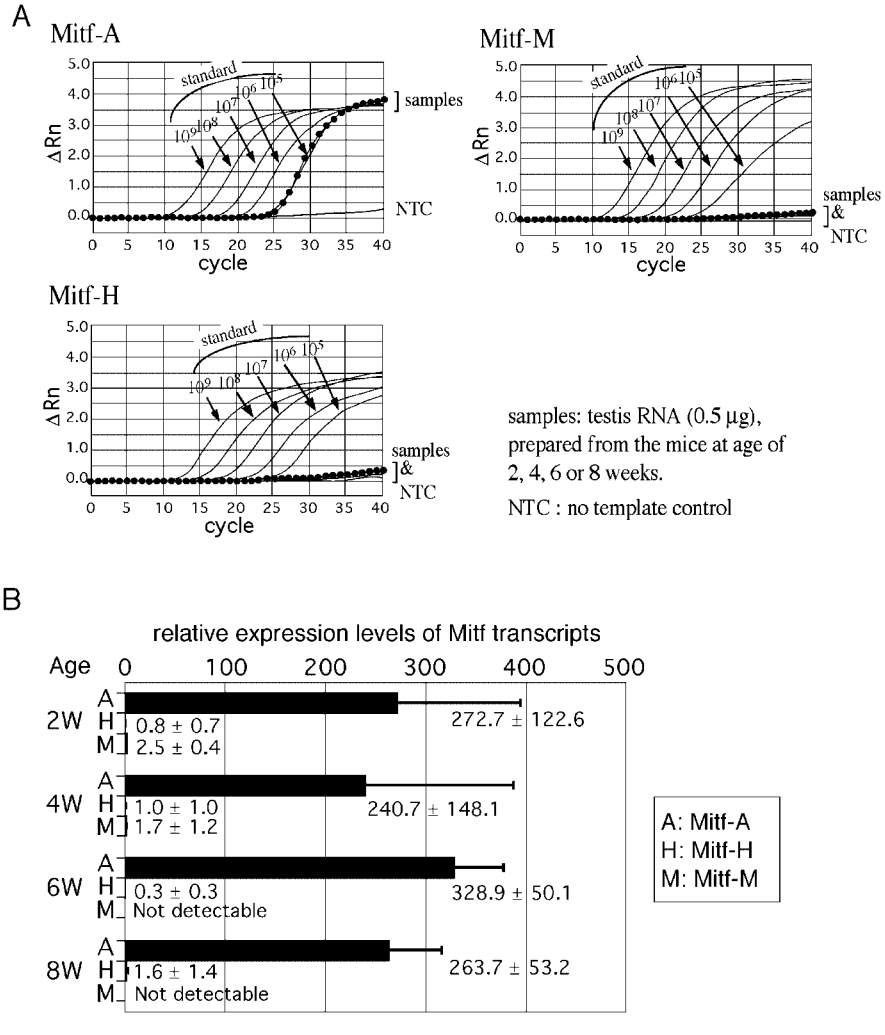

Fig. 3. **Real-time PCR analysis of Mitf transcripts in the mouse testis.** A: Quantitative analysis of exon 1 transcripts. Testis RNAs were prepared from postnatal mice at 2, 4, 6, and 8 wk of age. The panels show the standard curves for Mitf-A, Mitf-H, or Mitf-M RNA, together with the copy numbers of the given isoform RNA. Rn stands for normalized fluorescence measurement. The relative levels of transcripts containing each exon 1 sequence were similar at each of the four ages (2 wk; 4 wk; 6 wk; 8 wk). Note that only the data for the RNA sample at 8 wk are shown (closed circles); the data for other ages are shown in B, because all samples gave curves that largely overlapped one another. The curves for NTC (no template control) are also drawn. Shown are examples of three independent analyses. B: Relative expression levels of Mitf transcripts in the mouse testis during postnatal development. The results of three independent experiments are shown with standard deviations. The expression levels of Mitf-H and Mitf-M transcripts were below background.

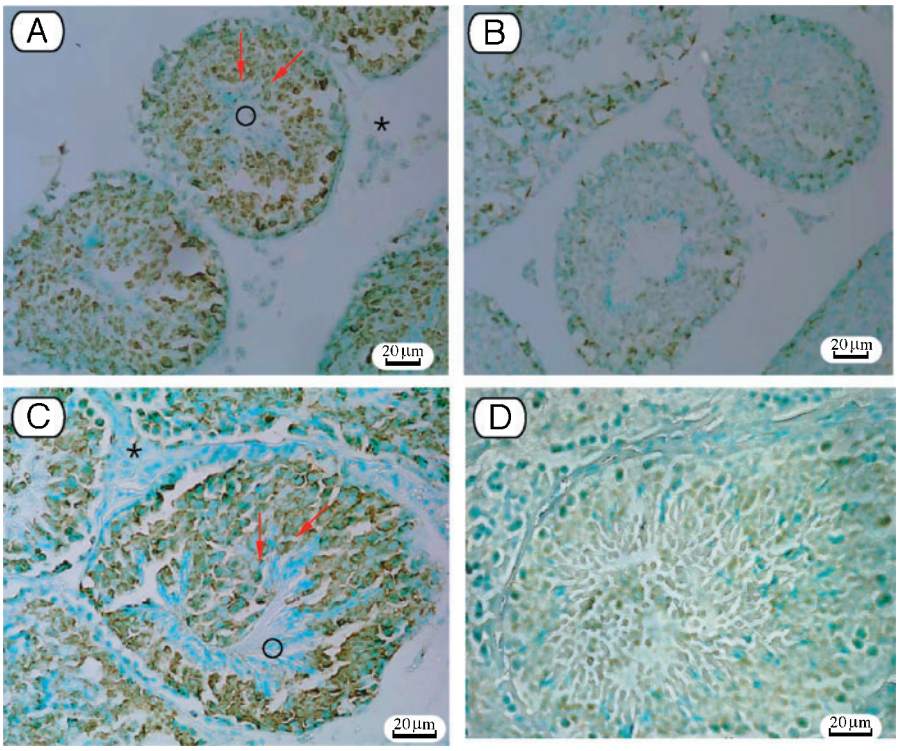

Fig. 4. **Expression of Mitf mRNA in the mouse testis.** Shown are tissue sections of mouse testes analyzed by *in situ* hybridization. Testes were isolated from wild type mice (A, B) and *Mitf*$^{mi\text{-}bw}$ mice (C, D). The integrity of the seminiferous tubules was slightly disturbed because the tunica albuginea testis was dissected before fixation. The tissue sections were hybridized with an anti-sense probe in the absence (A, C) or presence of a 100-fold excess of unlabeled probe (B, D). Signals that represent Mitf mRNA expression are detectable in spermatogonia, spermatocytes, and round spermatids (arrows). Note that Mitf mRNA is undetectable in elongated spermatids near the lumen (circles) and in somatic Leydig cells (asterisks).

under conditions that allowed us to amplify the 5′-ends of the cDNAs for Mitf isoforms, such as Mitf-H (*7*). Only Mitf-D cDNA was consistently amplified, indicating that Mitf-D is expressed in mouse testis (Fig. 2A). Mitf-D was initially identified in RPE cells and monocyte-lineage cells affected by *Mitf* mutations (*7*). Unexpectedly, we found base changes between C3H and BALB/c mice in the 5′-untranslated region of the Mitf-D mRNA that is encoded by exon 1D (Fig. 2B). In the previous study, Mitf-D cDNA and its genomic segment were derived from BALB/c mice (*7*). These base changes may represent polymorphism among mouse strains.

Expression of Mitf Isoform Transcripts in the Testis—In the mouse, primary spermatocytes initiate meiosis by 9 days of postnatal age, and spermiogenesis is initiated by 20 days (*30*, *31*). Testosterone production increases dramatically between 4 and 5 wk of age, and breeding starts by 6–7 wk (*32*). We therefore compared the expression levels of Mitf-A, Mitf-H, and Mitf-M transcripts in the testis during postnatal development (2, 4, 6, and 8 wk) by real-time PCR analysis (Fig. 3). The PCR primers were designed within a single exon 1 (exon 1A, 1H or 1M) to assess the level of transcription from each exon 1, as alternative splicing is very common in the testis (*24*, *33*, *34*). However, we were unable to include exon 1D transcripts for the real-time PCR analysis, because of the small size of exon 1D (25 bp). Accordingly, we measured the relative levels of transcripts containing the sequences of exon 1A, exon 1H or exon 1M. The standard curves with synthetic RNA indicate that the method employed can accurately measure the levels of Mitf-A, Mitf-H, and Mitf-M mRNAs (Fig. 3A). Under these conditions, similar levels of exon 1A transcripts were detected in RNA samples prepared from testes during the postnatal period (Fig. 3B). Notably, the levels of exon 1H transcripts were undetectable (near the baseline). As expected, exon 1M transcripts were also undetectable. These results indicate that the RNA preparations were not contaminated with genomic DNA. Likewise, only exon 1A transcripts were detected in RNA samples prepared from the testes of *Mitf*$^{mi\text{-}bw}$ mice during postnatal development (data not shown).

Mitf Expression in Male Germ Cells—To identify the cell types that express Mitf mRNA in the testis, we performed *in situ* hybridization analysis using a cRNA probe common to all Mitf isoform mRNAs (see Fig. 1A). The testes used were isolated from wild type and *Mitf*$^{mi\text{-}bw}$ mice at age 8 wk, because the difficulty in maintaining *Mitf*$^{mi\text{-}bw}$ mice has been reported (*15*). As a negative control, hybridization was performed in the presence of a 100-fold excess of the unlabeled cRNA probe. Expression of Mitf mRNA, most likely Mitf-D and/or Mitf-A, is detectable in spermatogonia, spermatocytes that undergo meiosis, and round spermatids with the haploid genome (Fig. 4, A and B). Importantly, its expression is not detected in elongated spermatids, which represent late-stage spermatids with remodeled and condensed chromatin, and in which transcription is mostly repressed (*24*). Elongated spermatids are present near the lumen of the seminiferous tubules. Mitf mRNA expression is also undetectable in diploid peritubular cells and Leydig cells, which are located in the regions between seminiferous tubules. The testes from 8-wk-old *Mitf*$^{mi\text{-}bw}$ mice displayed normal spermatogenesis and spermiogenesis (*12*) and a regular structure and thickness of the lamina propria of seminiferous tubules and normal morphology of the interstitial tissue (Fig. 4, C and D). There is no noticeable difference in the expression patterns and levels of Mitf mRNA between wild type and *Mitf*$^{mi\text{-}bw}$ mouse testes. In addition,

Mitf-D cDNA was preferentially amplified from testis RNA of *Mitf*$^{mi\text{-}bw}$ mice by 5′-RACE (data not shown).

DISCUSSION—Among the N-terminal regions of Mitf isoforms examined, only domain A of Mitf-A exhibited a noticeable transactivation activity in yeast cells. It is noteworthy that domain A shares significant amino acid identity with the N-terminus of TFE3, a member of a family of transcription factors with a bHLH-LZ structure (*35*, *36*). In addition, three consecutive portions, covering the entire domain A, are aligned to equivalent portions of cytoplasmic retinoic acid-binding proteins (*37*). In contrast, no transactivation activity was detected with domain H, domain B1b (the amino-terminal of Mitf-D) or domain M. Other approaches are required to assess the functions of those N-terminal domains. On the other hand, exon 1D, which codes for the 5′-untranslated region of Mitf-D mRNA, may confer tissue-restricted expression of the isoform on the *Mitf* gene, as expected from the fact that Mitf-D shares a primary structure with Mitf-E. Mitf-E is preferentially expressed in mast cells (*8*), whereas Mitf-D mRNA is undetectable in mast cells (*7*).

To the best of our knowledge, there have been no reports showing that *Mitf* mutant mice exhibit infertility or germ cell abnormalities. Thus, we do not know the physiological significance of Mitf expression in male germ cells. In this context, the classic *Mitf*^{mi} mutation recessively induces uterine inversion and reduces the number of newborns, but the testes and ovaries of homozygous *Mitf*^{mi} mice are morphologically normal (*38*). On the other hand, Mitf has been shown to be important in the regulation of c-kit expression in mast cells (*20*, *21*). In addition, Opdecamp *et al*. (*39*) have suggested that Mitf is dispensable for the initiation of c-kit expression in melanoblast precursors, but required for the maintenance of c-kit expression. Notably, in the mouse testis, c-kit is expressed on spermatogonia, early spermatocytes, and Leydig cells (*22*), but not on later spermatocytes and spermatids (*22*). Thus, Mitf mRNA appears to be coexpressed with c-kit only in spermatogonia and early spermatocytes, suggesting that Mitf might contribute to the maintenance of transcription of the *c-kit* gene in those types of germ cells. In contrast, expression of the *c-kit* gene may be independent of Mitf in Leydig cells, in which other factors are responsible for *c-kit* gene transcription.

Mitf-D exhibits cell-type restricted expression patterns; Mitf-D is expressed in RPE, macrophages, and osteoclasts affected by *Mitf* mutations, but not in other *Mitf*-target cells, such as melanocytes and mast cells (*7*). On the other hand, Mitf-H is expressed in those cell types (*7*) as well as in other types of cells that have been analyzed (*5*, *15*, *40*). Thus, an unexpected finding of the present study is that exon 1H transcripts representing Mitf-H mRNA were not detectable in testis RNA by real-time PCR analysis (Fig. 3). It is noteworthy that Mitf-D is expressed in the testis (Fig. 2) and that exon 1H and exon 1D are juxtaposed in the *Mitf* gene (Fig. 1A). Moreover, the genes must be transcribed at the lower temperature of 33°C in male germ cells. We, therefore, suggest that a testis-specific regulatory mechanism may lead to the repression of transcription from exon 1H while enhancing transcription from exon 1D; namely, certain *cis*-acting elements regulate transcription from exon 1H and from exon 1D in a mutually exclusive manner in male germ cells. This proposal is consistent in part with the report that aberrant Mitf mRNA is over-expressed in the testis of *Mitf*$^{mi\text{-}rw}$ mice, which carry a deletion of the genomic DNA segment containing exon 1H, exon 1D, and exon B1b (*3*, *14*, *17*). It is conceivable that aberrant Mitf transcripts are derived from exon 1A or cryptic initiation sites that are not normally used (see Fig. 1A).

The difficulty in obtaining a large number of *Mitf*$^{mi\text{-}bw}$ mouse offspring has been reported (*15*). However, there are no noticeable abnormalities in the testis, epididymis, or prostate, or in the numbers, shapes or motility of the sperm prepared from the epididymis of *Mitf*$^{mi\text{-}bw}$ mice (*12*). The present study confirms the normal morphology of their testes (Fig. 4, C and D). The expression levels of Mitf-A mRNA are reduced in the skin and eye of *Mitf*$^{mi\text{-}bw}$ mice, as judged by RT-PCR analysis (*15*), but the expression levels of exon 1A transcripts exhibit no noticeable changes during maturation of the testis derived from either wild type (Fig. 3) or *Mitf*$^{mi\text{-}bw}$ mice (data not shown). Probably, such a difference is in part due to the increased levels of alternate transcripts in this mutant mouse, which harbors the insertion of an L1 element in the downstream intron (*15*). We hypothesize that *Mitf*$^{mi\text{-}bw}$ mice may be susceptible to certain environmental factors that impair the hormonal or neural networks that regulate reproduction or mating behavior. We are currently searching for genes whose expression levels are altered in the brain of *Mitf*$^{mi\text{-}bw}$ mice.

In summary, the amino-terminus of Mitf-A, encoded by exon 1A, may be involved in modulating its transactivation function. Mitf-A and Mitf-D mRNAs are expressed in male germ cells, although we do not know the relative levels of these isoforms. Exon 1D and its upstream promoter may confer male germ cell-specific transcription on the *Mitf* gene, similar to the *M* promoter of exon 1M in melanocytes. The present study provides further evidence that multiple promoters of the *Mitf* gene generate differential expression patterns of Mitf isoforms as well as functional diversity.

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